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Short communication

The effect of temperature on the mucosal IgM antibody response to DNP-KLH in channel catfish (*Ictalurus punctatus*)

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ABSTRACT

Bath immersion remains a practical route for immunizing against disease in channel catfish; however research efforts in this area have revealed variable results when activating mucosal Ab responses with different antigens. This is likely due to a number of factors including the individual species, age of the fish, preparation of the immunogens, and differences in the overall dosage and the duration of exposure to vaccines. The current study sought to evaluate the effect of water temperature on the *in vivo* mucosal adaptive immune response in channel catfish to a protein-hapten antigen, DNP-KLH. Fish were bath immersed at different water temperatures and periodically evaluated over an eighteen week period for the development of serum and mucosal IgM antibodies to DNP-KLH using an indirect enzyme-linked immunosorbent assay. None of the temperature groups produced a serum antibody response; however there were detectable DNP-KLH specific IgM antibodies in the mucus starting at week eight. The extent of the mucosal antibody response and duration differed between the treatments. Our results show that there are intrinsic differences in the capacity to generate *in vivo* mucosal Ab responses in the skin at different water temperatures and the implications of these findings to channel catfish farming will be discussed.

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1. Introduction

Catfish farming remains number one among U.S. aquaculture commodities with 2015 and 2016 revenues of over $300 million a year. Several pathogens continue to disrupt the catfish industry and according to the last major census by the National Animal and Health Monitoring System [1], bacterial disease accounted for over 50% of food fish losses. As production continues to increase, the frequency of disease will only continue to rise. Add to this an increase in the regulation of chemical therapeutants and resistance to available antibiotics means that alternative methods of disease prevention are required. Vaccination against different fish pathogens has proven to be a valuable tool in disease management practices [2–4]. While it has been shown that many fish species generate antibodies to different antigens in their mucosal tissues, additional work is needed to gain insight into the basic mechanisms that stimulate and maintain adaptive immune responses in the mucosae of teleost fish [5–8]. Current protocols to immunize channel catfish have focused on bath immersion utilizing various immunogens. These studies have sought to optimize the conditions under which these immunizations occur, including the age of the fish, and the duration of exposure and dosage of antigens [2,9]. Other basic studies have sought to demonstrate the importance of water temperature on the *in vitro* and *in vivo* systemic adaptive cellular response to antigens [10,11]; however the temperature required at the mucosal tissues to generate an adaptive immune response has not been examined [12,13]. The current study sought to characterize the effect of water temperature on the *in vivo* ability to generate a mucosal IgM antibody response to the soluble DNP-KLH antigen.

2. Materials and methods

2.1. Fish and experimental conditions

Channel catfish were reared at the Harry K. Dupree Stuttgart National Aquaculture Research Center in Stuttgart, Arkansas, USA. A total of twelve adult catfish, weighing between 1.0 and 1.5 kg, were equally stocked into each of three 200 L tanks that received filtered well-water and aeration from submerged air stones that were

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maintained at different water temperatures (15, 23 and 30 °C). The ambient temperature (23 °C) well-water was cooled (15 °C) or heated (30 °C) by use of a heat pump or portable tank heater(s) regulated with NEMA 4× digital temperature controllers (Aqua-Logic, San Diego, CA). Dissolved oxygen and temperature were measured daily using an YSI Pro20 dissolved oxygen meter (Yellow Springs, Ohio). The average water temperature during the study for each of the three tanks was 15.1 ± 1.2, 23.2 ± 1.1 and 30.0 ± 1.4 °C during a twenty-week period. Fish were offered pelleted catfish feed to satiation three days a week (35% protein, 2.5% fat; Delta Western, Indianola, Mississippi). The animal care and experimental protocols were approved by the Stuttgart National Aquaculture Research Center Institutional Animal Care and Use Committee and conformed to Agricultural Research Service Policies and Procedures.

2.2. Immunization, serum and mucus sampling

Fish were acclimated to the different water temperatures one week prior to immunization. DNP-KLH (EMD Millipore, Germany) was prepared as a 20 mg/ml stock solution in laboratory-grade water. To immunize the fish, the well-water to the three tanks was turned off and the water level was lowered to 100 L and the DNP-KLH stock solution was added to form a bath immersion (50 μg/ml). After 30 m the well-water in each tank was turned on and allowed to fill to normal levels. Each of the four fish from the three groups was sampled every four weeks for 18 weeks, with the exception of two (30 °C) fish that had to be euthanized at 16 weeks for a non-disease related reason. For sampling, fish were anesthetized (MS-222, Western Chemical, Ferndale, WA); blood was collected using a 21-gauge needle from the caudal vein, allowed to clot overnight at 4 °C and then centrifuged at 10000 x *g* for 10 min using an Eppendorf Minispin; the serum was removed and stored at -20 °C until needed. A blood sample was taken from each fish a day before the DNP-KLH immunization. Skin mucus samples were collected by gentle scraping of the dorso-lateral surface using a plastic cell scraper with enough care to avoid abrading the skin. The mucus was placed into 5.0 ml of 10 mM Tris-Cl buffer (pH 7.0) and the homogenate was vortexed briefly and centrifuged to collect the insoluble mucus fraction at the bottom of the tube. The total protein content of the soluble mucus fraction was estimated using the Pierce Coomassie plus assay kit (ThermoFisher, Waltham, MA) with bovine serum albumin (Sigma Aldrich, St. Louis, MO) as the standard. Absorbance was read at a wavelength of 595 nm with a BioTek Synergy H1 plate reader operating under Gen5 software (Winooski, VT). In a few instances the soluble mucus fraction required concentration using an Amicon Ultra-0.5 centrifugal filter unit (EMD Millipore, Billerica, MA) in order to achieve an average concentration (200 μg/ml).

2.3. Western blot and indirect ELISA

Gel electrophoresis (1-D SDS) was conducted on samples of catfish serum and mucus fractions using 10% TGX stain-free gels and buffers of the mini-protean system (Biorad, Hercules, CA). We electrophoresed 10 μg of serum proteins and 20 μg of the soluble mucus proteins onto an SDS gel with a pre-stained gel marker (Biorad, Hercules, CA) and then stained using Simple Blue Safe (ThermoFisher, Waltham, MA) or transferred onto nitrocellulose membranes utilizing the Transblot system (Biorad, Hercules, CA). For western blot analyses transfers were first blocked for 1 h at room temperature with 1× PBS with 0.05% Tween-20 (PBST) and 5% milk. The membrane was washed two times in PBST and then incubated with the anti-channel catfish IgM mouse monoclonal antibody 9E1 [14] at 1:500 dilution in blocking solution for 1 h; then washed two times in PBST and stained with sheep α-mouse IgG-HRP polyclonal antibody (1:5000 GE Healthcare, Pittsburgh, PA) for 30 m. The chemiluminescent signals were developed using Pierce ECL plus Western Blotting Substrate according to the manufacturer's protocol (ThermoFisher, Waltham, MA) and visualized using a Biorad ChemiDoc XRS + gel system operating under Image Lab 5.2.1 software.

An indirect ELISA was used to measure DNP-KLH specific serum and mucosal IgM antibodies. Prior to conducting these experiments; we first established the amount of DNP-KLH required to bind to and generate a consistent OD signal using a DNP-KLH reactive pAb (A6430, ThermoFisher, Waltham, MA). Pierce Reactbind 96-well plates (ThermoFisher, Waltham, MA) were coated with 100 μl of 15 μg/ml of DNP-KLH resuspended in a sodium bicarbonate buffer for 1 h at room temperature. Plates were then rinsed three times with PBST and then incubated for 1 h in blocking solution (PBST with 5% milk). One hundred μL of serum (1:100) or mucus (1:2) were added to the plate and then further serially diluted out to 1:1600 for serum or 1:32 for mucus in 1× PBS on the horizontal axis and incubated at room temperature for 1 h. Plates were rinsed as above and 100 μl of the monoclonal antibody 9E1 was added at 1:500 dilution in blocking solution. An anti-trout IgM monoclonal antibody was used as an isotype control [15]. After 1 h of incubation at room temperature, plates were washed with PBST and 100 μl of sheep α-mouse IgG horseradish-peroxidase polyclonal Ab was diluted 1:5000 in blocking solution and incubated for 30 m at room temperature. Plates were rinsed three times with PBST, and 100 μL of 1-Step Ultra TMB-ELISA substrate solution (ThermoFisher, Waltham, MA) was added. The peroxidase reaction was stopped after 20 min with 100 μL of 3M H_2SO_4 and read spectrophotometrically at 490 nm with a BioTek Synergy H1 plate reader operating under Gen5 software (Winooski, VT).

2.4. Statistics

Differences between the serum and mucus DNP-KLH specific antibody levels at different time points within each temperature group were evaluated using a Dunnett's multiple comparisons test, and overall differences between temperature groups were evaluated using a two-way ANOVA, and different time points within each temperature group used a Tukey's multiple comparisons test. Probabilities of <0.05 were considered statistically significant. All statistics were performed using Prism 7.0 (GraphPad, San Jose, CA).

3. Results and discussion

Before examining the production of DNP-KLH specific antibodies in the skin of channel catfish; we first sought to evaluate the level with which IgM was present and detectable in the soluble mucus fraction sampled from the skin. Previous reports on teleost immunoglobulins of mucus secretions of the intestine and skin have determined there are nanogram (ng) levels of immunoglobulins as compared to the serum component with milligram (mg) amounts [7,16]. However, we and others had previously shown that an indirect ELISA could detect IgM antibodies both in the mucus and in the tissue culture medium of catfish skin explants during an immune response [17–19]. SDS gel analysis was run on serum and mucus fractions from a single adult channel catfish (Fig. 1). The initial analysis showed the differential protein banding patterns between the serum (lane 2) and mucus (lane 3) samples when developed with Coomassie stain (Fig. 1). The subsequent transfer of the SDS gel and probing for catfish IgM using the 9E1 monoclonal antibody resolved a dominant signal in the serum and much less so in the mucus of the expected size of the IgM H chain (lanes 4 and 5, Fig. 1). In this experiment, twice the amount of mucus as serum was

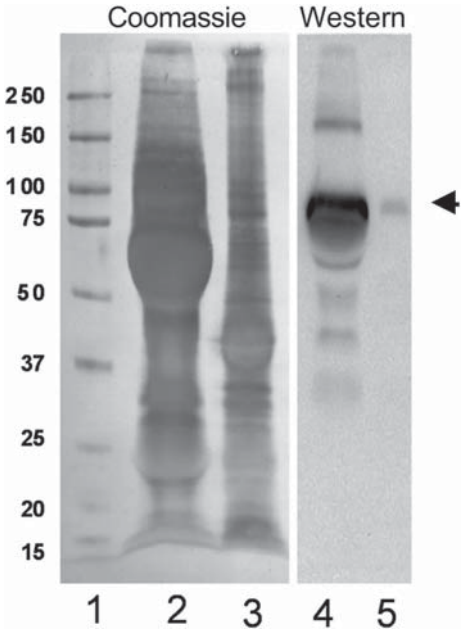

Fig. 1. Analysis of immunoglobulin M in the serum and mucus of channel catfish. Coomassie gel and Western blot of serum (lanes 2 and 4) and mucus (lanes 3 and 5), respectively are indicated. The arrow identifies the IgM heavy chain. The Western C marker (lane 1) was used to estimate molecular mass (kDa).

loaded onto the gel in order to resolve an IgM signal; the overall result is however consistent with what others have shown wherein the levels of catfish mucosal IgM can vary greatly under normal or disease conditions [20,21].

To begin to understand the *in vivo* kinetics of antigen specific antibody development in the serum and mucus; fish held at different water temperatures were sampled every four weeks over an eighteen week period after a bath immersion with DNP-KLH. The antigen was used because it had been shown to be a sound T-dependent antigen in teleost fish [12,22]. An indirect ELISA was conducted to evaluate DNP-KLH specific serum IgM antibody titer (Fig. 2). Preliminary absorbance values (the relative level of DNP-KLH IgM antibodies) showed that individual fish had very little if any pre-existing serum IgM antibody that bound to DNP-KLH. The 15, 23 and 30 °C grouped fish had mean absorbance values of 0.124 ± 0.005, 0.121 ± 0.009 and 0.130 ± 0.01, respectively. An evaluation of DNP-KLH-specific antibodies using the serum from other non-immunized experimental fish showed similar absorbance values to DNP-KLH (data not shown). There was no significant increase in DNP-KLH specific IgM antibodies during the eighteen weeks among the different temperature groups from that observed in their pre-immune serum. These data would therefore suggest there was no direct effect from the bath immersion on the production of DNP-KLH-specific systemic IgM antibodies, and is consistent with the idea that mucosal-based stimulation can be independent of the systemic immune system [5,7,12].

To next determine the outcome of IgM antibody production in the skin to DNP-KLH the mucus was also evaluated using the same indirect ELISA. There was no pre-immune sampling of the mucus; however at week 4 the mean absorbance's (0.103 ± 0.009, 0.100 ± 0.006 and 0.095 ± 0.01) among the 15, 23 and 30 °C groups were similar to those of the pre-immune serum (Fig. 3). In contrast to the serum, the 15, 23 and 30 °C mucus did then begin to show differences beginning at week 8 with mean absorbance values of 0.153 ± 0.015, 0.249 ± 0.095 and 0.210 ± 0.016, respectively. The 15 and 23 °C groups were significantly higher ($P < 0.05$) than those observed among the individual temperature groups at week 4. By week 12 only the 23 °C and 30 °C groups had mean absorbance values (0.169 ± 0.009 and 0.165 ± 0.01) that remained significantly higher ($P < 0.05$) than that observed at week 4. By week 16 all groups had absorbance values similar to week 4. Interestingly, the 23 °C group resulted in a much more robust DNP-KLH response at week 8 when evaluating the mean absorbance of the two high responders (0.319 ± 0.09) as compared to the two low responders (0.178 ± 0.002, Fig. 3). Due to this variability there was no significant difference overall to the earlier time point, however individual variability in the systemic and mucosal antibody responses to different Ag(s) is often observed [17,20,21].

Nearly thirty years since the first experiments were done to understand the differences between the systemic and mucosal immune systems of teleost fish; we continue to try and understand and ultimately optimize *in vivo* mucosal responses to antigen [5,7,23]. The leukocyte populations necessary to activate a T-dependent antibody response were present in the skin of catfish as evidenced by the detection of DNP-KLH specific mucosal IgM antibodies. Further evaluation of these antibody responses showed a significant difference in the amount of antibody produced at weeks 8 and 12 between the colder (15 °C) and warmer (24 and 30 °C) temperature groups (Fig. 3), with the fish held at the lower temperature generating less mucosal antibody. The effect of temperature on the development of primary antibody-secreting cells in catfish was first evaluated through the use of an *in vitro* cell culture

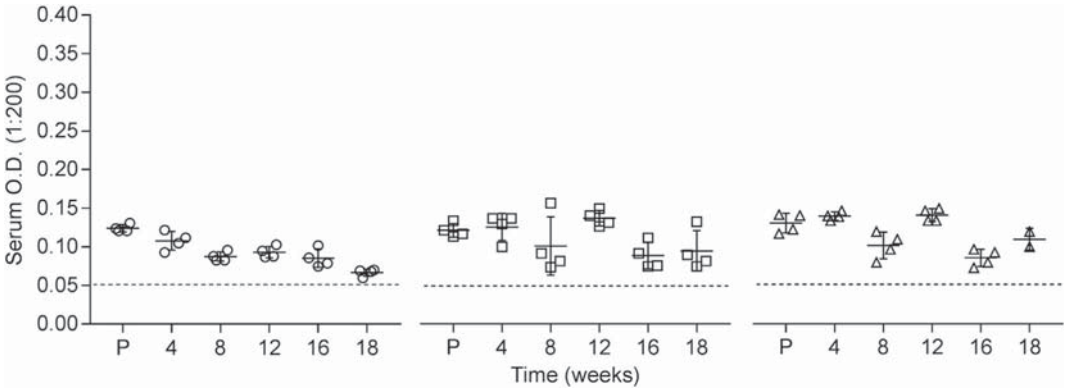

Fig. 2. The *in vivo* development of DNP-KLH specific systemic IgM antibodies of channel catfish at different water temperatures. The temperature groups are from left to right as follows: 15 °C (circles), 23 °C (squares) and 30 °C (triangles). The serum IgM antibody titer (1:200) is the reciprocal of the maximum dilution at which we could reliably detect binding of antibody to DNP-KLH. The mean absorbance ± SD for each group is shown as a horizontal line. The dashed line represents a background absorbance observed using an isotype control. (P) Pre-immune serum.

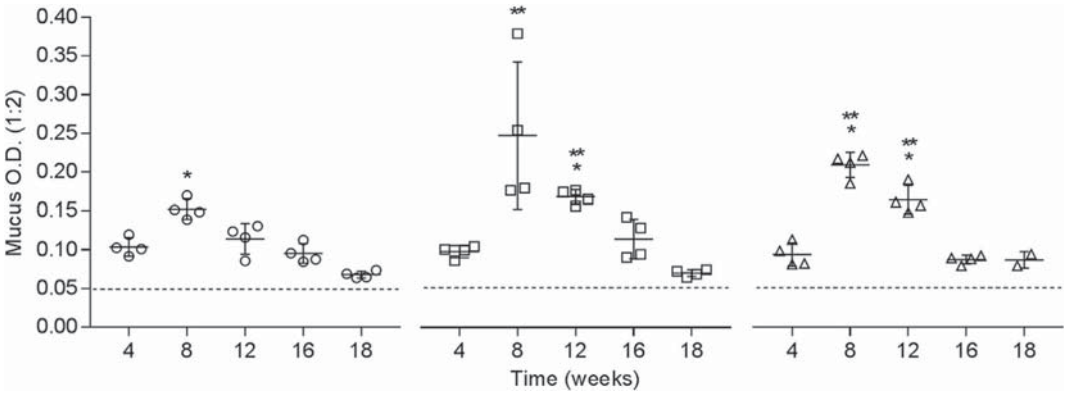

Fig. 3. The *in vivo* development of DNP-KLH specific mucosal IgM antibodies of channel catfish at different water temperatures. The temperature groups are labeled the same as in Fig. 2. The mucosal IgM antibody titer (1:2) is the reciprocal of the maximum dilution at which we could reliably detect binding of antibody to DNP-KLH. The mean absorbance ± SD for each group is shown as a horizontal line. The dashed line represents background absorbance observed using an isotype control. An asterisk denotes a significant difference; $P < 0.05$ when week 4 of each temperature groups was compared to every other time point within each individual temperature group. Asterisks denote significant differences; $P < 0.05$ when weeks 8 and 12 of the 23 °C and 30 °C groups were compared to the 15 °C group.

system, where systemic leukocytes were adjusted to temperatures ranging from 17 to 32 °C prior to their exposure to a T-dependent antigen. This pioneering work of Miller and Clem [10] demonstrated that the requirement for an effective T-dependent response was conditional on the temperature to which the leukocytes were held upon antigen activation. A second study using a similar *in vitro* assay sought to evaluate the efficiency of a T-dependent response through an *in vivo* water temperature decrease [11]. This work showed that with a rapid decrease of *in vivo* water temperature (23 °C–11 °C in 24 h), the *in vitro* response to antigen was suppressed for up to four weeks. These results in part confirm that the colder temperature in the present study limited the development of a DNP-KLH specific mucosal antibody response.

As stated earlier, there is an ongoing potential for the catfish mucosae to be exposed to opportunistic pathogens during the production cycle. One such pathogen is *Flavobacterium columnare*, a gram negative bacterium that causes significant disease through the formation of biofilms to the gill and skin of catfish and illustrates the importance for optimizing protocols for mucosal vaccination [24,25]. Current vaccines recommend bath immersion during the fry stage, but there is some debate as to how beneficial they are this early on during the development of the catfish immune system [26,27]. An increase in the use of hybrid channel catfish and in single batch systems suggests that the optimal time to vaccinate is at the fingerling stage prior to transport into the production systems. Our results show that a minimum of 4–8 weeks is required to develop mucosal antibodies, therefore warmer water temperatures during this time will be critical in the development of the humoral response. A thirty-five year survey of water temperatures in production systems in the Mississippi Delta demonstrated that a mean temperature at or above 23 °C occurred between the middle of May through the beginning of October [28]. Therefore depending on the time of year the effectiveness of these immunizations could be directly affected by the ambient water temperature. The results presented here highlight the importance of temperature on the development of mucosal antibody responses and further underscores the need for additional research to optimize vaccination practices within the catfish farming industry.

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